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APPLICATION OF RECOMBINANT DNA TECHNOLOGY TO METHANE BIOSYNTHESIS

by

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Cooperative Agreement No. CR810340

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16. ABSTRACT

A project was conducted to clone the genes encoding the polypeptide subunits of the enzyme methyl-coenzyme M methyl-reductase (methyl CoM-reductase). The experimental approach was to purify the enzyme, produce antibodies against the enzyme, and use these antibodies to screen Escherichia coli colonies for clones that synthesized antigens with which the anti-methyl CoM-reductase antibodies reacted. The E. coli strains contained plasmids or were preinfected with bacteriophages that had been constructed by in vitro DNA recombinant techniques to contain fragments of either M. thermoautotrophicum or M. vannielii genomic DNA's. The expectation was that the E. coli clones that reacted with the anti-methyl CoM-reductase antibodies would contain cloned methanogen DNA sequences encoding part or all of the methyl CoM-reductase polypeptides. However, this technique frequently generated false positive signals. Most of the study period was used in improving the technology to decrease the number of antifactually positive signals and in screening and analyzing positive clones that ultimately were found to contain none of the desired genes.

Although the specific goal of cloning the genes encoding methyl CoMreductase has yet to be reached, the experiments completed has produced valuable information describing the structures and organization of methanogen genes and the structure of methanogen messenger RNA's.

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FOREWORD

The U.S. Environmental Protection Agency is charged by Congress with protecting the Nation's land, air, and water systems. Under a mandate of national environmental laws, the agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. The Clean Water Act, the Safe Drinking Water Act, and the Toxic Substances Control Act are three of the major congressional laws that provide the framework for restoring and maintaining the integrity of our Nation's water, for preserving and enhancing the water we drink, and for protecting the environment from toxic substances. These laws direct the EPA to perform research to define our environmental problems, measure the impacts, and search for solutions.

The Water Engineering Research Laboratory is that component of EPA's Research and Development program concerned with preventing, treating, and managing municipal and industrial wastewater discharges; establishing practices to control and remove contaminants from drinking water and to prevent its deterioration during storage and distribution; and assessing the nature and controllability of releases of toxic substances to the air, water, and land from manufacturing processes and subsequent product uses. This publication is one of the products of that research and provides a vital communication link between the researcher and the user community.

This publication provides the results of experiments designed to determine the basic structure of genes in the methane-producing microorganisms (methanogens) which inhabit, and are responsible for a major degradative activity, in waste-water treatment facilities. Methanogens catalyze the final step in the conversion of biological waste materials to the inocuous and valuable biofuel, methane. Development of novel methanogens and/or genetic manipulation of existing organisms to improve their efficiency in waste-water treatment and to increase the range of their detoxification capabilities is a realistic goal of modern biotechnology. To reach this goal and assess the practical benefits of genetically-engineered methanogens in waste-water treatment facilities will require a thorough knowledge of the enzymology, molecular biology and genetics of these very unusual microorganisms. This publication describes results of research, sponsored by the EPA, aimed at generating a body of basic information on which future decisions on applying biotechnology to waste-water treatment and handling can be based.

Francis T. Mayo, Director
Water Engineering Research Laboratory

ABSTRACT

A project was conducted to clone the genes encoding the polypeptide subunits of the enzyme methyl-coenzyme M methyl-reductase (methyl CoM-reductase). The experimental approach was to purify the enzyme (initially from Methanobacterium thermoautotrophicum, and subsequently from Methanococcus vannielii), produce antibodies against the enzyme, and use these antibodies to screen Escherichia coli colonies for clones that synthesized antigens with which the anti-methyl CoM-reductase antibodies reacted. The E. coli strains contained plasmids or were preinfected with bacteriophages that had been constructed by in vitro DNA recombinant techniques to contain fragments of either M. thermoautotrophicum or M. vannielii genomic DNA's. The expectation was that the E. coli clones that reacted with the anti-methyl CoM-reductase antibodies would contain cloned methanogen DNA sequences encoding part or all of the methyl CoM-reductase polypeptides. However, this technique frequently generated false positive signals. Most of the study period was used in improving the technology to decrease the number of artifactually positive signals and in screening and analyzing positive clones that ultimately were found to contain none of the desired genes. Because enzyme purification and antibody production were very time consuming and artifactual results were being obtained, alternative approaches were investigated and experiments were undertaken to identify the sequences in methanogen DNA's that act as regulatory signals for gene expression. This information will be needed in the next stage of this project; the construction of DNA molecules containing methyl CoM-reductase sequences that can be manipulated to direct the synthesis of the enzyme when reintroduced into methanogens.

Although the specific goal of cloning the genes encoding methyl CoM-reductase has yet to be reached, the experiments completed have produced valuable information describing the structures and organization of methanogen genes and the structure of methanogen messenger RNA's.

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SECTION 1

INTRODUCTION

Anaerobic Fermentation and Methanogenesis

Anaerobic digestion has been used for almost a century for waste stabilization, waste disposal, and particularly in underdeveloped countries and rural areas, for production of the fuel methane.

Methane production by anaerobic fermentation involves the conversion of organic materials at modest temperatures, ambient pressures, and nearly neutral pH to methane and carbon dioxide in the absence of exogenous electron acceptors such as oxygen, nitrate, and sulfate through a complex series of microbial interactions. During the process, which is promoted almost exclusively by mixed bacterial populations, the yield of new microbial cells is relatively small. Most of the substrates' chemical energy ultimately resides in methane despite the fact that an equal number of carbon dioxide molecules are formed when fermentation is proceeding in a balanced, steady-state mode. For example, ignoring the small amount of substrate that is used to replace cellular biomass and to provide cellular maintenance energy, the gross stoichiometry of the methane fermentation of glucose can be approximated by:

$$C_6H_{12}O_6(aq) = 3CH_4(g) + 3CO_2(g)$$

The standard Gibbs free energy and enthalpy changes for this conversion under physiological conditions (pH 7, 25°C, unit activities) per mole of glucose are about -418 and 131 kJ, and the mass and energy contents of the methane, expressed as fractions of the glucose converted, are about 27 and 95 percent respectively . Thus the thermodynamic driving force is large, the exothermic energy loss is small, and energy is transferred at a higher energy density to a simple gaseous fuel. Methane produced is easily separated from the aqueous environment, and concomitant with methane production, the mass of the waste organics used as substrates is significantly reduced.

Microbiological Process Limitation

Present-day anaerobic digestion processes yielding methane have been established by allowing events that occur in sediments, in the rumen, and in other natural anaerobic environments to take place within a constructed container. There are many variants of such containers, and much attention has been paid to engineering aspects of the process. By contrast, biological design has been lacking. In no respect does the understanding of methanogenesis in anaerobic digesters begin to approach the level characteristic of established fermentation procedures, such as those used to manufacture ethanol, antibiotics, and other pharmaceutical compounds. Process control (in the sense of monitoring and adjusting the process to allow biological difficulties to be anticipated and

controlled) is essentially absent. The process in industrial practice is empirical, and apart from regulation of parameters such as temperature, pH, and substrate addition, is uncontrolled. Consequently, it is unreliable when judged by present-day standards in other fermentation industries. The basis for this lack of process control is the shortage of information describing the regulation of microbial metabolism that occurs within a digester. This information is needed before process control can be logically developed. The overall flow of different materials through anaerobic fermenters has frequently been studied, and many of the microbial species involved in the digestion process have been isolated. The information now required is how exactly these species interact and what are the critical regulatory steps in the digestion process. How are the microbial interactions and regulatory steps affected by substrate and environmental changes? To underline our limited knowledge in this area, Zeikus (1) concluded in a recent review that the mechanism for regulating enzyme activity levels in methanogens is not known.

Biology and Molecular Biology of Methanogens

The study of methanogens has been impeded by their extreme sensitivity to oxygen; they require redox potentials below -330 mV for growth, and only recently have techniques been developed by which methanogens can easily be cultivated as pure cultures in the laboratory (2,3). The advent of these techniques has facilitated a rapid expansion in the study of the biology of methanogens and the biochemistry of methane biogenesis. Results obtained to date indicate that methanogens form an extremely diverse group (2,4,5 are reviews). They span the full range of prokaryotic morphological types and have DNA's with base contents ranging from 27.5% to 52% G+C. In fact, it has been proposed that methanogens are not true prokaryotes but are representives of a third biological kingdom called the Archaebacteria (2,6). This kingdom, which includes extreme halophilic and acido-thermophilic micro-organisms in addition to the methanogens, was proposed because its members have several properties radically different from both prokaryotic and eukaryotic species. Archaebacteria have unique structural subunits in their cell envelopes and lipids (7). Their RNA and protein synthesizing systems are resistant to most of the antibiotics that inhibit these processes in prokaryotes and eukaryotes (3,8,9). Their DNA-dependent RNA-polymerases are very different from those of classical prokaryotes (10), and comparative analyses of the sequences of tRNA's and rRNA's have provided convincing evidence that whereas the different archaebacterial types are related to each other, they are only very distantly related, in evolutionary terms, to current prokaryotic and eukaryotic species (2). Recently it has been shown that some archaebacterial tRNA genes contain intervening sequences, introns, a property generally associated with eukaryotic but not prokaryotic species (11). Our studies have focussed on cloning and analysis of polypeptide encoding genes. We have been able to clone fragments of DNA from several different methanogens that function in E. coli to complement auxotrophic mutations in this eubacterial species (12-15). The long-term goals of our research program are to obtain an understanding of the structure and activity of enzymes directly involved in methane biogenesis and to create improved methanogenic species for use in conversion of biomass to methane. The most abundant enzyme in methanogens is methyl-coenzyme M methyl-reductase which catalyzes the final reaction in methane biosynthesis. The availability of procedures to purify this enzyme coupled with its obvious major importance in

methane production led to our decision to concentrate our studies on this enzyme and to clone its encoding genes. This report documents our progress toward this goal.

SECTION 2

CONCLUSIONS

The specific goal of cloning the genes that encode the subunit polypeptides of methyl-coenzyme M methyl-reductase has not yet been reached. We have obtained a number of clones able to synthesize antigens that interact with antibodies raised against the subunits of methyl CoM-reductase. These clones must be screened further to determine whether any of them contain the desired recombinant DNA molecules. The experiments undertaken to isolate and characterize methanogen mRNA's have provided the first detailed description of archaebacterial mRNA structures and have generated the RNA substrates for development of an *in vitro* translation system. Analyses of cloned methanogen genes, which complement auxotrophic mutations in *E. coli* and *B. subtilis*, have resulted in publications containing the first DNA sequences of methanogen-derived, protein-encoding genes. In addition, we have isolated and characterized the first methanogen insertion element (ISM1), and we have provided the first description of regulatory elements used in expression of methanogen genes. Although the methyl CoM-reductase genes of *M. vannielii* are not yet isolated, we feel that the technical problems encountered can be overcome. Nevertheless, the results of our research to date have already provided a firm foundation for future applications of recombinant DNA technology to methane biogenesis.

SECTION 3

RECOMMENDATIONS

The research undertaken during the period of this contract has established basic knowledge of the structure of methanogen genes. It is now clear that cloning and expression of genes directly involved in methane biogenesis in novel microbial species is a practical goal. The DNA sequences already cloned should be further characterized. It will be necessary to determine directly the amino acid sequence of the purified subunit polypeptides of methyl-coenzyme M methyl-reductase. Once this information is available, it can be used to design synthetic DNA probes and to correlate with experimentally determined DNA sequences. The gene libraries constructed during the current period represent major resources that could be used as sources of other important methanogen genes.

The limiting reaction in an anaerobic, methanogenic fermenter is the availability of hydrogen. Hydrogen uptake is catalyzed by hydrogenases, and the recommendation is therefore made that genes encoding hydrogenases in methanogens should also be subjected to intense study. Manipulation of hydrogenase activities would be a major asset in designing and applying practical process control to waste digestion. Manipulation of enzyme activities cannot be logically approached without a thorough knowledge of the structure and regulation of expression of the genes that encode the enzymes of interest. The experiments described in this report and recommended for future research are designed to obtain this type of basic information for practical application in anaerobic waste treatment.

SECTION 4

PROCEDURES AND RESULTS

Purification of Methyl-coenzyme M methyl-reductase (Methyl CoM-reductase).

The terminal step in methane biogenesis involves the reduction of a methyl group bound to the cofactor, co-enzyme M. This reaction is catalyzed by methyl CoM-reductase, an enzyme which accounts for approximately 10% of the total cellular protein of methanogens. The holoenzyme form of methyl CoM-reductase, which has been isolated from a number of methanogenic species, is a complex of 3 differently-sized polypeptide subunits ($\gamma_2\ \beta_2\ \alpha_2$) with a combined molecular weight of approximately 300,000 daltons. The enzyme is bound to a Ni containing cofactor known as F_{430}. We have purified this enzyme from M. thermoautotrophicum, a thermophilic methanogen (year 1) and M. vannielii, a mesophilic methanogen (years 2 and 3) by following published procedures (16, 17).

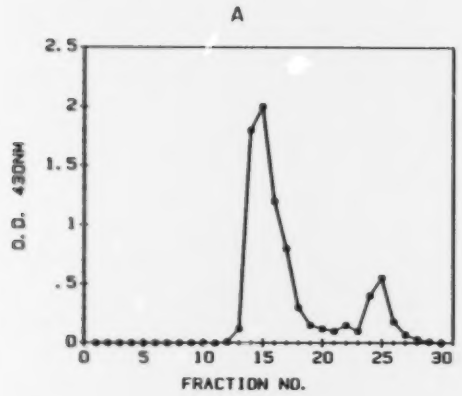
The enzymes involved in methane biogenesis are extremely oxygen sensitive and therefore initial purification steps were conducted in an anaerobically-maintained glove box. M. thermoautotrophicum and M. vannielii cultures were grown autotrophically in bottles of minimal medium, with a 20% CO_2: 80% H_2 v/v atmosphere maintained at 15 p.s.i. Cells (10 g) were harvested in air-tight, stainless-steel centrifuge bottles under a H_2 atmosphere, resuspended in buffer A (0.05 M Tris, pH7.0, 10 mM β-mercaptoethanol) and gassed with H_2 to remove all traces of O_2. M. vannielii cells are osmotically sensitive and therefore lysis occurred rapidly in buffer. Lysing cells of M. thermoautotrophicum, however, required use of the French press (12,000 p.s.i.). Eluants from the French press were collected, under a stream of H_2, into stainless-steel centrifuge tubes. Cell lysates, of both strains, were clarified by centrifugation at 33,000 xg for 30 minutes and the supernatants (adjusted to 30 mg of protein per ml with buffer A) were stored under a H_2 atmosphere at -20°C.

Extracts were further purified by DEAE-cellulose (DE-50) column chromatography. This procedure separated the extract into 3 components (A,B and C) which, when mixed together, reduced the methyl group of methyl-CoM to methane *in vitro* (16). Component A contained the F_{420}-reducing hydrogenase, component B was a heat-stable low molecular weight cofactor and component C was the methyl CoM-reductase, the enzyme of interest. To perform anaerobic column chromatography, the anaerobic chamber was appropriately modified to allow its use for spectrophotometric analyses and collection of column fractions. The columns and fraction collector were placed inside the anaerobic chamber, a U.V.-recording spectrophotometer, pump and refrigeration unit were located adjacent to, but outside the anaerobic compartment. Conduits were built which allowed anoxic cooling fluid or column eluates to be circulated through the refrigeration unit or through the spectrophotometer and returned to the

anaerobic environment. The methyl CoM-reductase enzyme absorbs at a wave length of 430nm and therefore it was possible, by monitoring adsorption at 430 nm to follow enzyme elution from columns.

Anaerobic extracts (treated with DNase to reduce viscosity) were applied to DEAE-cellulose columns (2.5 X 20 cm) pre-equilibrated with anaerobic buffer A and 4 ml fractions were collected at a rate of 20 ml per hr. Components A, B and C were eluted in a batchwise manner. The column was first washed with 160ml of buffer A; all of component A eluted. Increasing the salt concentration of buffer A to 0.1 M eluted component B from the column in 100ml of buffer. Elution of component C, including NADPH oxidizing enzymes was obtained by washing the column with 0.32 M salt in buffer A. Components A and B were stored under H_2 at -20°C. Fractions containing component C were pooled, concentrated to 15-20 ml by ultrafiltration and dialyzed against anaerobic buffer A containing 0.8 M salt. At this stage, component C was not O_2 labile and could be purified under aerobic conditions. The final step in purification of component C used aerobic gradient sievesorptive chromatography at 4°C. A DEAE-sephadex A-25 column (2.5 x 35 cm) was equilibrated with buffer A containing 0.1M salt. Prior to sample application, a 50-90 ml gradient of 0.1-0.4 M salt in buffer A was loaded onto the column. The sample (15-20 ml) was then applied, the column washed with 1M salt in buffer A and 6 ml fractions collected at a rate of 10 ml per hr. The absorbance at 430nm was monitored by directing column eluates through a spectrophotometer. The abundance of the enzyme and the known sizes of the subunit polypeptides made identification of methyl CoM-reductase in the fractions by SDS-polyacrylamide gel electrophoresis (PAGE), a practical assay. Enzymatic assays, namely methane generation from methyl-CoM, were not routinely used. A typical spectrophotometric elution profile of the enzyme, after its first passage through the DEAE sephadex column, together with the corresponding PAGE analysis is shown in Figure 1. Fractions containing 430 nm absorbing material are shown by SDS-PAGE analysis to contain the three enzyme subunits, indicating a good correlation between the two assays (Figure 1). It should be noted that at this stage of the purification contaminating polypeptides were still detectable. Fractions containing methyl CoM-reductase were pooled and concentrated to 20 ml by ultrafiltration. Further purification of methyl CoM-reductase was obtained by successive passage of enzyme-containing fractions through at least three more DEAE-sephadex columns, preloaded with decreasing salt gradients, until the enzyme was almost free of detectable contaminating proteins (Figure 2). Yields of purified methyl CoM-reductase varied from 1-5 mg per preparation. Purified enzyme was stored at -20°C. In one purification, the culture of M. thermoautotrophicum was grown in the presence of ^{63}Ni to radioactively label the methyl-CoM-reductase. The ^{63}Ni co-purified with the enzyme as expected from previous documented evidence that Ni is tightly bound to this enzyme.

To increase the purity of the enzyme preparations, several modifications to the published DEAE-sephadex chromatography procedure were necessary. Changes were made to the salt concentration of the sample and elution buffer, in the volume and molarity range of the preformed salt gradients and in the rate at which the column was run. Decreasing the volume of the salt gradients to 50 ml for each column run and employing a 0.2-0.3 M linear gradient in the final two columns improved the final enzyme purity. None of the enzyme preparations however were completely free of small amounts of contaminating protein (< 1% of



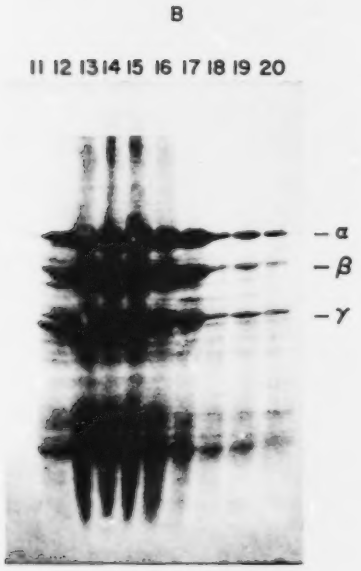


Figure 1. Analysis of eluates from the first DEAE-sephadex column. A. Spectrophotometric profile of column eluates. B. SDS-PAGE analysis of column eluates. Fraction numbers are indicated at the top of the gel. Each lane of the gel contains 0.4% of the protein in each fraction. Protein was stained with coomassie blue. The separated γ, β and α subunits are indicated at the right of the gel.

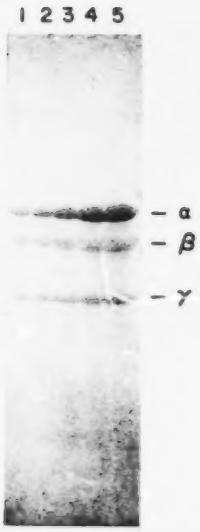


Figure 2. SDS-PAGE analysis of purified M. vannielii methyl CoM-reductase. Lanes 1, 2, 3, 4, and 5 contain 5, 10, 15, 20, and 25 μg of enzyme, respectively. Protein was stained with coomassie blue. The separated γ, β and α subunits are indicated at the right of the gel.

total protein). Staining of samples of the enzyme following SDS-PAGE with the highly sensitive silver stain always demonstrated the presence of traces of contaminating proteins.

Production and evaluation of anti-methyl CoM-reductase antisera.

Antisera were raised against methyl CoM-reductase by immunization of young, white, New Zealand rabbits with purified antigen. The most purified enzyme preparations obtained from M. thermoautotrophicum or M. vannielii were employed to produce antisera against these complexes of three polypeptides. Standard vaccination techniques were used. Preimmune serum was taken from each rabbit, prior to vaccination, and stored at -20°C. The enzymes (200-500 µg) were emulsified in complete Freund's adjuvant and injected into a hind leg. Following a 10 week period for the primary immune response, 100 µg of antigen (prepared in incomplete Freund's adjuvant) was injected, subcutaneously along the back muscle, to boost antibody production. Sera were taken from the ear at weekly intervals and assayed by a standard enzyme-linked immunosorbent assay (ELISA) using alkaline phosphatase labeled anti-rabbit goat IgG as the secondary antibody. High-titer sera were pooled and the IgG fraction purified by chromatography through a DEAE-affinity gel blue (Biorad) column. Re-assay of these antibodies against M. thermoautotrophicum and M. vannielii methyl CoM-reductase showed that the preparations had ELISA titers of 1/1600 and 1/3200, respectively.

The enzyme preparations used for vaccination contained additional methanogen proteins, albeit in very small amounts and therefore it was possible that the antibody preparations obtained might have been contaminated, particularly if the co-purified, contaminating proteins were strongly immunogenic. Thus in screening gene libraries for clones producing methyl CoM-reductase protein using these antisera as probes, false positive clones might have been obtained (see below). In later vaccinations, the three subunits of methyl CoM-reductase, from both M. vannielii and M. thermoautotrophicum, were further purified from contaminating proteins by SDS-PAGE. Antibodies raised against the separated subunits also had the advantage that positive signals could be related directly to the gene encoding the polypeptide used as the antigen. A potential disadvantage of the separated polypeptides was that they were denatured during the SDS-PAGE and therefore may not have had all the same antigenic determinants as the native enzyme.

For production of antisera against the three individual subunits of M. vannielii methyl CoM-reductase, the enzyme subunits were disaggregated by exposure to SDS and separated on preparative SDS-PAGE gels. Regions of the gel containing the subunits were identified by staining and excised from the gel slab. Approximately 250 µg of each subunit was prepared. Gel slices were homogenized and passed through an 18 gauge needle to obtain fine particles of acrylamide containing the antigen. Rabbits were vaccinated with these gel slurries; 150 µg and 50 µg of protein were used in the primary and booster injections, respectively. These antigen preparations were not emulsified in Freund's adjuvant since acrylamide served as an adjuvant. Sera were collected weekly after the booster injection. Assays of antibody titers by ELISA showed that high levels of anti-γ and anti-β antibodies were obtained after only one week and were maintained for one month. In contrast, only a low level of anti-α

antibodies was produced. In an attempt to improve the titer of the serum containing anti-α antibodies, the rabbit was given a second boost (50 μg of protein), eight weeks following the first boost. Assays of the serum then collected showed much higher titers. High titer sera preparations obtained from vaccination with the individual subunits of methyl-CoM-reductase were assayed by ELISA. The titers were anti-γ (1/5000), anti β (1/10,000) and anti α (1/5000).

The specificities of the antisera preparations raised against the individual subunits were evaluated by Western blots. Protein extracts of M. vannielii, M. thermoautotrophicum and E. coli, together with purified methyl CoM-reductases were subjected to SDS-PAGE. The separated polypeptides were transferred, electrophoretically, onto nitrocellulose paper using a trans-blot apparatus. Blots were incubated overnight in the appropriate antisera, diluted 1/100, and washed to remove unbound antibody. Immune complexes formed on the nitrocellulose were allowed to react with either ^{125}I-labeled sheep-anti-rabbit IgG or ^{125}I-protein A. The radioactive complexes so formed were detected by autoradiography. An autoradiogram of a Western blot of M. vannielii methyl CoM-reductase subunits probed with the antisera preparations containing either anti γ, and anti β or anti α antibodies is shown in Figure 3. The blot shows that antibodies raised against each purified subunit are strictly specific for the polypeptide used as the immunogen; there was no binding to other polypeptides in M. vannielii lysates. The high molecular weight polypeptide, in the lysate of M. vannielii which also reacted with the anti-γ sera, (Figure 3A, lane 1), was not present in other blots and most likely represented a precursor or non-denatured form of the γ subunit. Antibodies raised against the individual subunits of M. thermoautotrophicum methyl CoM-reductase were also shown to recognize only the polypeptides used as the antigen. Furthermore, antibodies raised against the native forms of methyl CoM-reductase, from both organisms, reacted only with the three subunit polypeptides in lysates from the homologous strain.

Western blotting analyses were also undertaken to determine if the antibodies raised against the methyl CoM-reductase from M. thermoautotrophicum or M. vannielii recognized and bound to the equivalent subunits of methyl CoM-reductase in extracts of other methanogens. The results showed that antibodies produced against the M. thermoautotrophicum enzyme also bound to the γ, β and α polypeptides of M. vannielii methyl CoM-reductase and vice versa. Furthermore, this cross-reactivity extended beyond M. thermoautotrophicum and M. vannielii as anti-methyl CoM-reductase antibodies prepared against this enzyme from these two organisms also recognized and bound to the analogous methyl-CoM-reductase polypeptides in crude extracts of four other methanogenic species, namely; Methanococcus voltae, Methanococcus thermolithotrophicus, Methanobrevibacter smithii and Methanosarcina barkeri. These observations demonstrate that the methyl CoM-reductase enzyme from at least these six methanogens must have conserved amino acid sequences and therefore most probably similar secondary and tertiary structures. This cross-reactivity is potentially very useful, since the antibody preparations produced to date could be used to identify clones containing methyl CoM-reductase genes from other methanogens, thus avoiding the task of preparing the enzyme from all species of interest.

Unfortunately, Western blotting experiments also revealed that all antisera preparations contained antibodies which reacted, to various extents, with E.

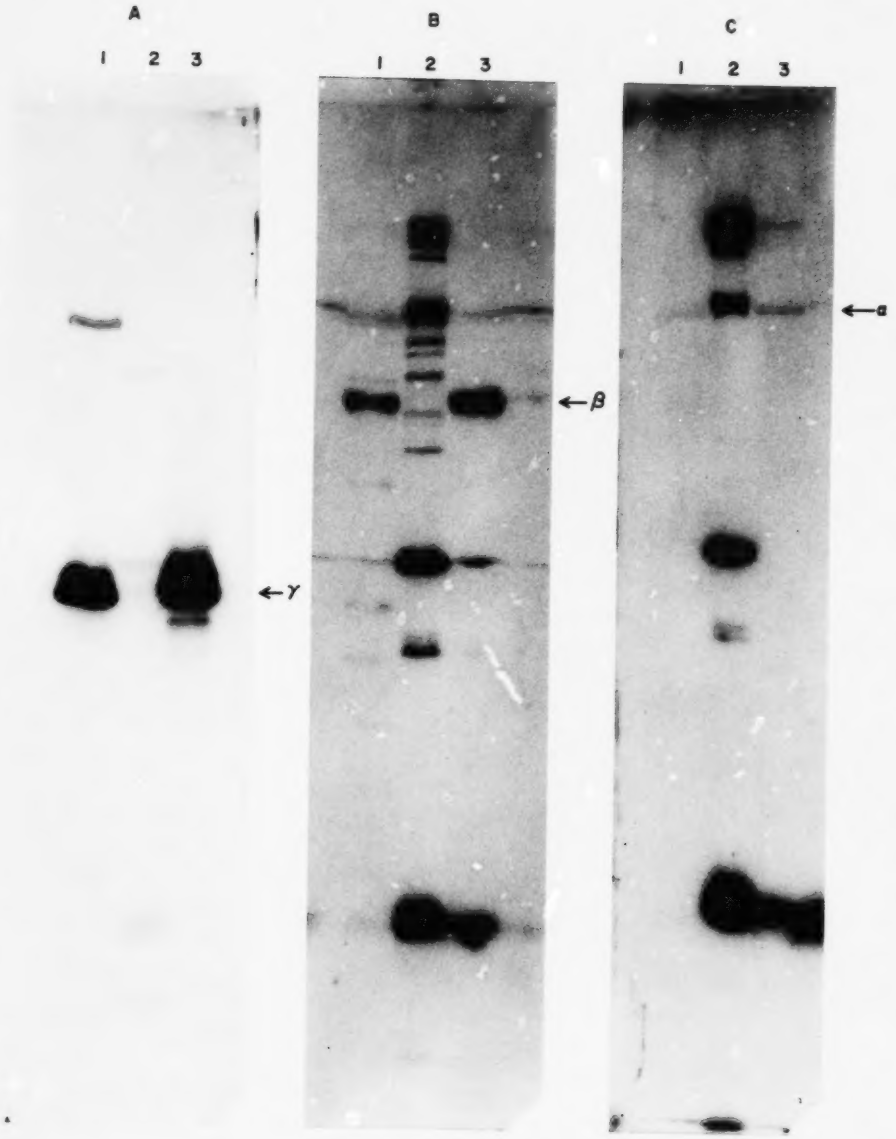


Figure 3. Western blot of M. vannielii methyl CoM-reductase polypeptides. Polypeptides were electrophoresed in a 15% SDS-PAGE gel: lanes 1, M. vannielii lysate; lanes 2, E. coli lysate and lanes 3, purified M. vannielii methyl CoM-reductase. Separated polypeptides were transferred to nitrocellulose and the triplicate filters probed with anti-γ (A), anti-β (B) and anti-α (C) antibody preparations. Immune complexes were detected with ^{125}I-protein A. The autoradiogram is shown. Positions of the subunit polypeptides which reacted with the individual antisera preparations are indicated.

coli proteins (see Figure 3, lane 2 for an example). The presence of anti-E. coli antibodies probably reflects environmental exposure of the rabbits, used to produce antisera, to E. coli organisms. The anti-E. coli antibodies in the antisera preparations interfered with the screening for methyl CoM-reductase antigens synthesized in E. coli by creating a high background. Steps were therefore taken to remove these contaminating antibodies. An E. coli lysate was immobilized on a solid membrane support, (nitrocellulose) and incubated with the antibody preparation. As most of the DNA cloning procedures employed λ bacteriophage-based vectors, lysates were produced from λ-infected E. coli cultures to obviate problems with anti-λ antibodies in rabbit antisera. Such λ antibodies were detected in Western blotting experiments. Although the majority of anti-E. coli antibodies were removed after several successive adsorption treatments we were never able to completely remove all of the anti-E. coli antibodies.

The strong positive signals for methyl CoM-reductase obtained by Western blots of methanogen lysates were encouraging, however, based on the assumption that E. coli cells infected with a lytic recombinant phage (plaque) or carrying a recombinant plasmid (single colony) might synthesize only very low amounts of methyl CoM-reductase antigen, it was deemed important to evaluate the sensitivity of the various methods available for detecting antigen:antibody (Ag:Ab) complexes before full scale screening of gene libraries with antibody probes was undertaken. A preparation of purified methyl CoM-reductase was serially diluted and an aliquot of each dilution spotted onto a nitrocellulose filter. The filter was submerged in the antiserum being tested and, following an incubation period to allow complete Ag:Ab interaction, the filter was removed, washed and the presence of immune complexes determined. Initially, two related procedures for this determination were evaluated. In both cases the antigen-rabbit-antibody complex was bound by sheep anti-rabbit antibody. The presence of the secondary antibody was then detected by its being either covalently linked to ^{125}I or linked to horseradish peroxidase. ^{125}I-labeled complexes were detected by autoradiography whereas the presence of horseradish peroxidase was detected by adding a chromogenic substrate. In later determinations, ^{125}I-protein A, which binds directly to the Fc region of antibody molecules, was similarly evaluated. The ^{125}I based assays were found to be approximately 10 fold more sensitive (recognizing as little as 10 pg of antigen) than the enzyme based assay. In the majority of experiments, ^{125}I-protein A was used. Dilutions of rabbit antiserum were therefore chosen which could detect less than 0.1 ng of methyl CoM-reductase antigen concentrated in a plaque-sized spot on a nitrocellulose filter, to screen either plaques on lawns of E. coli or single colonies of E. coli.

Construction of Genomic Libraries of M. vannielii and M. thermoautotrophicum DNA.

A genomic library is defined as a population of recombinant DNA molecules which is large enough to contain multiple copies of all genes of interest. The number of DNA molecules required for a complete gene library is dependent on the size of the organism's genome and the average size of the individual restriction fragments of DNA cloned into the hybrid recombinant molecules. Since the number of clones required to produce a gene library decreases as the average size of the insert DNA increases, we initially decided to employ λ-based vectors which

allow insertion of large (10-30 kb) DNA restriction fragments. For example, the genome size of M. thermoautotrophicum has been estimated to be 1.1 X 10^9 daltons (1.7×10^6 base pairs). If the average size of genomic restriction fragments is set at 20 kb, a representative λ-based gene library of M. thermoautotrophicum DNA should be comprised of less than a 1000 clones. We decided to use the λ-based vectors (λ1049 and λCh30) to clone 15-30 kb fragments of M. thermoautotrophicum DNA. Unfortunately there were no enzymatic techniques available to break open cells of this species and therefore the only techniques available for cell lysis incorporated a physical rupturing procedure such as sonication, French press or cryoimpacting. Using these procedures, most of the genomic DNA obtained was sheared into small fragments less than 20 kb in size. It was impossible to obtain sufficient amounts of high molecular weight DNA for cloning into the λ vectors. Some recombinants were obtained, but it was unlikely that a representative library was produced. To overcome this problem, libraries were produced using the plasmid vectors pUC8 and pMF4. Vector pUC8 contains multiple cloning sites within the lacZ gene which allows cloning of DNA fragments generated by a large range of restriction enzymes. Expression of genes cloned into pUC8 can be controlled by inducing transcription from the lacP promoter with isopropyl-thiogalactoside (IPTG). Later cloning experiments also employed plasmid pMF4. Very small DNA fragments (50-200 bp) were cloned into this vector at a site located between the amino-terminal portion of the λ c1 gene and the carboxyl terminal region of the lacZ gene. If cloning produced an inframe, open reading frame (probability of 1 in 6), a polypeptide would be synthesized which comprised the amino acid sequence specified by the cloned methanogen DNA sandwiched between the amino terminus of λ c1 and the carboxyl terminus of lacZ. As the number of clones obtained using either pUC8 or pMF4 vectors was sufficient to theoretically constitute representative genomic libraries, detection of polypeptides containing methyl CoM-reductase sequences should therefore have been possible using the antibody probes to screen these gene libraries.

The problem of obtaining large DNA molecules for λ cloning was bypassed when cloning experiments were directed towards producing gene libraries of M. vannielii. Cells of this species could be lysed easily by the addition of the ionic detergent SDS. This gentle procedure totally avoided mechanical cell disruption and therefore reduced random breakage of DNA. Genomic DNA fragments of 50 to 100 kb were readily obtained. Using this DNA, genomic libraries of M. vannielii were constructed employing the bacteriophage vectors λ Ch35 and λgt11. The vector λgt11 can be used as an expression vector. The λ-based recombinant molecules were packaged in vitro into complete λ particles and used to infect appropriate E. coli hosts to produce plaques. Immunoscreening of λgt11 recombinant libraries has been used successfully in a number of laboratories to isolate genes of interest. Efforts were therefore concentrated on constructing λgt11 based genomic libraries using different restriction digests of M. vannielii DNA (i.e. HaeIII, HincII and EcoRI). Insertion of foreign DNA into the EcoRI site of the lacZ gene carried by λgt11 facilitated expression of both hybrid proteins and other intact methanogen proteins encoded by cloned DNA fragments. This approach eliminated the need for the cloned DNA to contain transcription and translation regulatory signals. A problem in expressing foreign DNA in E. coli is that fusion polypeptides are sometimes specifically and rapidly degraded (18). The lon protease of E. coli appears to be responsible for this proteolysis and therefore the strains used for plating

recombinant phages were lon⁻ mutants, to ensure that fusion proteins would be stable.

The protocol used to generate λgt11 expression libraries is schematically represented in Figure 4. In brief, genomic DNA from M. vannielii was partially digested with HaeIII, HincII or EcoRI restriction enzymes to generate fragments with an average size of 5 kb. The restriction fragments from each digestion were treated with EcoRI methylase to protect internal EcoRI sites from cleavage in later steps. In order to facilitate the cloning of HaeIII and HincII fragments into the EcoRI site of λgt11, EcoRI linker oligonucleotides were ligated to the blunt ends of the molecules. HaeIII and HincII fragments were converted to EcoRI fragments by digestion with EcoRI and excess linkers removed from the DNA fragments by passage through sephadex G-75 columns. DNA fragments were finally ligated to EcoRI cleaved, alkaline phosphatase-treated λgt11 DNA and the resulting recombinant molecules packaged in vitro. The restriction deficient, modification proficient (r^-m^+) strain E. coli Y1088 was infected with the λ particles and plated on medium containing IPTG and X-gal, a chromogenic indicator of lacZ expression. Since DNA cloned into the EcoRI site of λgt11 inactivates the lacZ gene of this vector, plaques are white on X-gal plates. Strain Y1088 infected with the vector λgt11 alone yields blue plaques. The gene libraries generated contained approximately 5 X 10^4 plaque forming units of which 60-80% gave white plaques; these were recombinant phages. The recombinant λ libraries were finally amplified to produce higher titer viral stocks which were screened with the antibody probes. Plasmid gene libraries of M. vannielii DNA were also produced using the plasmid vector pUC8. M. vannielii DNA was double digested with HindIII/EcoRI, HindIII/BamHI, HindIII/PstI and HindIII/SalI and ligated to pUC8 DNA cleaved with the same combination of restriction enzymes. The plasmid libraries so generated (1 x 10^5 to 1 x 10^6 clones) contained greater than 80% recombinant DNA molecules. Based on the large number of clones obtained and the high ratio of recombinant to vector molecules, the λgt11 and pUC8 libraries were expected to contain copies of all the DNA sequences in the genome of M. vannielii.

Immunoscreening of genomic libraries

Over the last two years of this project M. vannielii became the organism of choice in experiments in our laboratory designed to develop a genetic exchange system for methanogens. Since future studies of gene regulation will demand a gene transfer technology, it was decided to concentrate on screening gene libraries of M. vannielii for cloned methyl CoM-reductase genes with antibody probes. Plaques produced by infection of E. coli with λ recombinant phages or colonies of E. coli containing plasmid-based recombinant molecules were screened for the presence of antigens which could bind anti-CoM-reductase antibodies.

The three λgt11 banks were screened separately with antisera preparations containing anti-γ, anti-β and anti-α antibodies using the method outlined in Figure 5. In the primary screening approximately 10^4 plaques were screened per plate. By using this screening method positive plaques from both the HaeIII and EcoRI gene libraries were identified with the anti-γ antibody probe (Figure 6A). Positive plaques were excised from the agar and the phage in the plaque purified until every phage in the population yielded a positive signal with the antiserum (Figure 6B). The phages which gave the positive signals from the HaeIII and

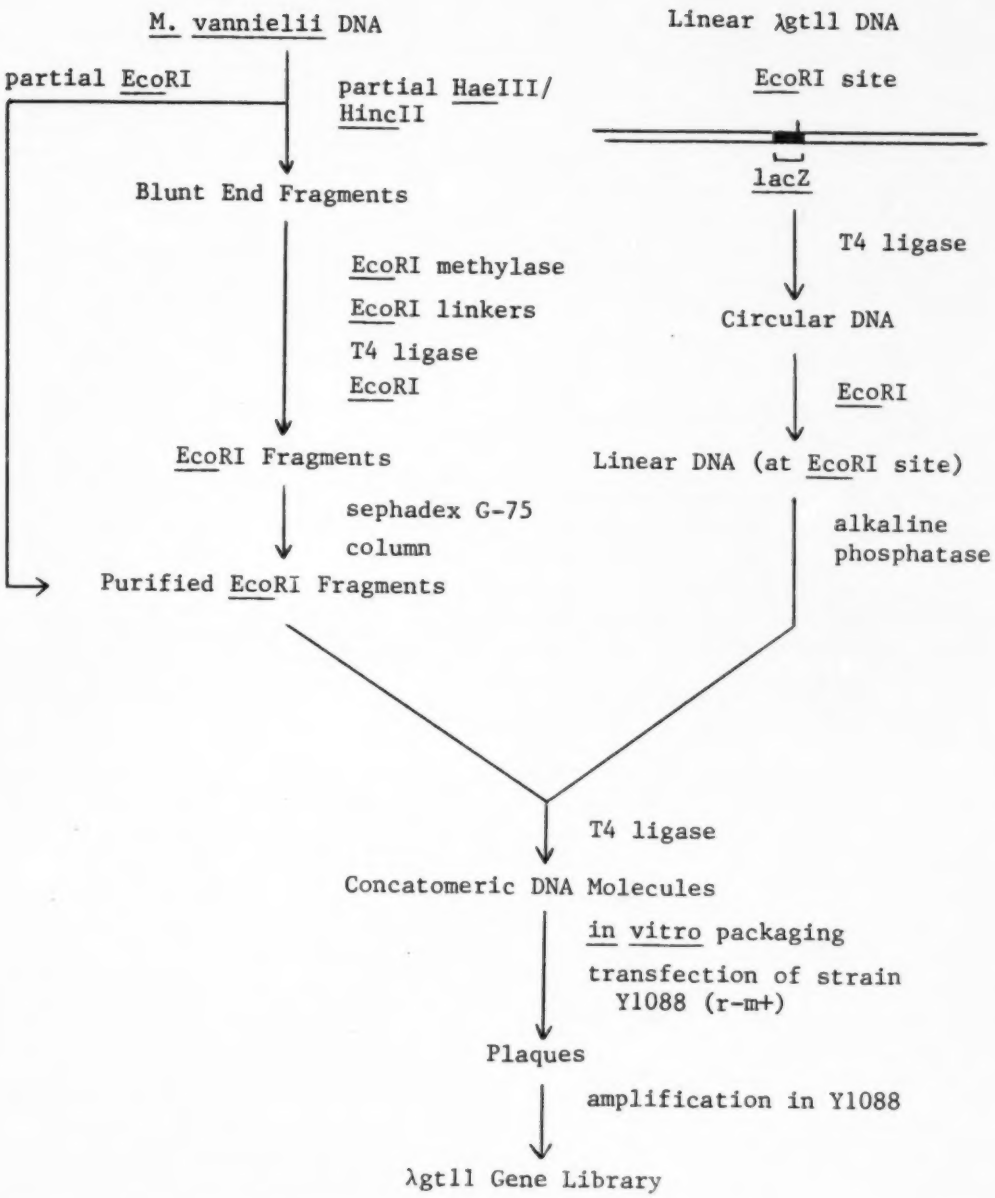


Figure 4. Cloning strategy used to construct the λgt11 recombinant DNA libraries.

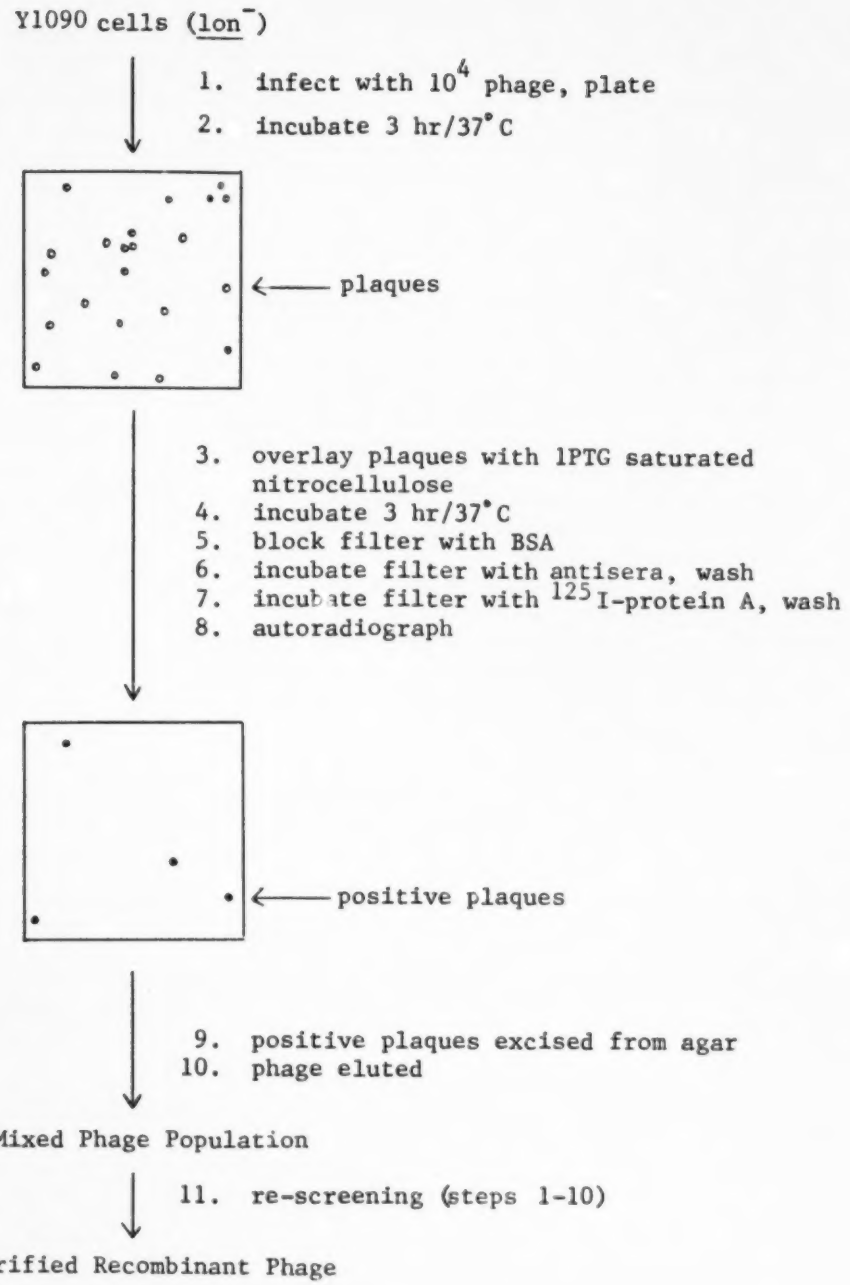

Y1090 cells (lon⁻)
1. infect with 10⁴ phage, plate
2. incubate 3 hr/37°C
plaques
3. overlay plaques with IPTG saturated nitrocellulose
4. incubate 3 hr/37°C
5. block filter with BSA
6. incubate filter with antisera, wash
7. incubate filter with 125I-protein A, wash
8. autoradiograph
positive plaques
9. positive plaques excised from agar
10. phage eluted
Mixed Phage Population
11. re-screening (steps 1-10)
Purified Recombinant Phage

Figure 5. Procedure used to screen the λgt11 gene libraries.

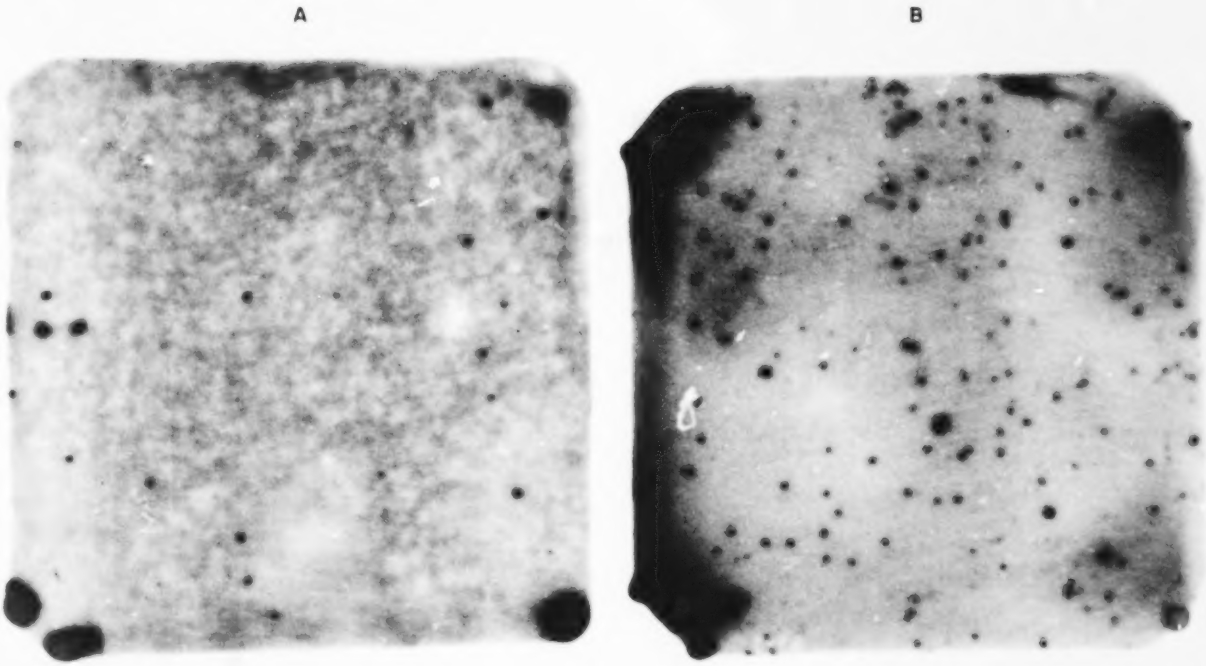


Figure 6. Autoradiogram of plaques screened with an antibody probe. A. Positive plaques from the primary screening of the λgt11 HaeIII library with the anti-γ antisera preparation. Fifteen of 10,000 plaques gave positive signals. B. Re-screening of purified recombinant phage. Every plaque yielded a positive signal. Dark regions at two of the corners on each filter represent the reaction of purified methyl-CoM-reductase with anti-γ Ab's.

EcoRI gene libraries were designated λgt-9γ and λgt-8γ, respectively. Positive plaques were not detected in any of the λgtll libraries using antisera containing either anti β or anti-γ antibodies. Further screening of these gene libraries with the antisera raised against the complete methyl CoM-reductase (MR) enzyme identified four more different, positive clones in the HaeIII gene library namely; λgt-MR-3A, λgt-MR-6A, λgt-MR-7A and λgt-MR-8A.

The E. coli libraries containing pUC8 based recombinant plasmid molecules were screened in a similar fashion. White colonies (harboring recombinant plasmids) were patched and grown on plates overlayed with a nitrocellulose filter saturated with IPTG. Cells which bound to the filter were lysed to release antigen and the filters screened with antisera as before (Figure 5). In primary screenings, four different colonies expressing antigen which reacted with antibodies in the anti-γ antisera were detected, namely; pUC8-γ1, pUC8-γ2, pUC8-γ3 and pUC8-γ4. Three different positive clones, pUC8-α1, pUC8-α2 and pUC8-α3 were isolated with the anti-α sera. Positive clones were not detected when the colonies were screened with anti-β antisera. Table 1 is a listing of all the positive clones isolated.

Analysis of positive clones.

Phages from plaques which contained antigen that bound anti-methyl CoM-reductase antibodies were produced as high titer stocks (10^{11}-10^{12} plaque forming units per ml). Similarly, plasmid DNA was purified from those colonies which synthesized an antigen recognized by either the anti γ or anti-α antisera. Restriction enzyme analyses were performed on both phage and plasmid DNA's to determine the size and restriction pattern of the cloned DNA. The EcoRI restriction patterns of the λgtll positive clones detected with the antibodies prepared against the methyl CoM-reductase complex are shown in Figure 7. The sizes of insert DNA's in all the positive clones isolated are listed in Table 1. Southern blotting of restriction digests of M. vannielii genomic DNA confirmed that the cloned DNA's had, in fact, originated from the genome of this organism.

Southern blotting experiments, were also used to determine the sequence relationships of the cloned DNA fragments. The result of one such experiment where λgt-8γ DNA was employed as a DNA probe is shown in Figure 8. It can be seen that λgt-8γ DNA hybridizes to a 0.8 kb fragment in both λgt-MR-3A and λgt-MR-7A DNA's (lanes 2 and 4, respectively) and also to a 1.6 and 1.95 kb fragment of λgt-MR-7A DNA (lane 4). Since the 0.8 kb and 1.95 kb fragments were also present in an EcoRI digest of M. vannielii DNA (lane 7) these two EcoRI fragments must be natural products of EcoRI digestion of the M. vannielii genome. On the basis of both restriction enzyme and Southern blotting analysis it was clear that several of the independently constructed phages did in fact contain common and overlapping restriction fragments; a result which was expected since partial restriction digests were used in the construction of the λgtll recombinant phages. These results were encouraging demonstrating that antibodies raised against methyl CoM-reductase facilitated the consistent isolation of the same fragment of methanogen derived DNA's.

The isolation of several additional unrelated phages was, however, also a concern in that it was clear that not all of these would contain the desired

TABLE 1 ANALYSIS OF POSITIVE CLONES

Phage/Plasmid	Reaction of phage/plasmid specified Ag with Ab preparations				Sizes (Kb) and type* of cloned methanogen fragment(s)
	anti-methyl CoM-reductase	anti-γ	anti-β	anti-α	
λgt-8γ	+	+	-	-	0.8,1.95
λgt-9γ	+	+	-	-	2.05
λgt-MR-3A	+	+	-	-	0.8, 0.9, 1.2, 2.1
λgt-MR-6A	+	-	-	-	0.55, 1.9, 2.0
λgt-MR-7A	+	+	-	-	0.8, 1.6, 1.95
λgt-MR-8A	+	-	-	-	0.95, 3.0
puc8-γ1	+	+	-	-	0.65 (HindIII/PstI)
puc8-γ2	+	+	-	-	0.75 (HindIII/PstI)
puc8-γ3	+	+	-	-	3.4 (HindIII/BamHI)
puc8-γ4	+	+	-	-	1.6 (HindIII/SalI)
puc8-α1	+	-	-	+	0.04 (HindIII/EcoRI)
puc8-α2	+	-	-	+	1.1 (HindIII/EcoRI)
puc8-α3	+	-	-	+	0.16 (HindIII)

*Fragments listed for the phage DNA are the EcoRI digestion products of the insert DNA.

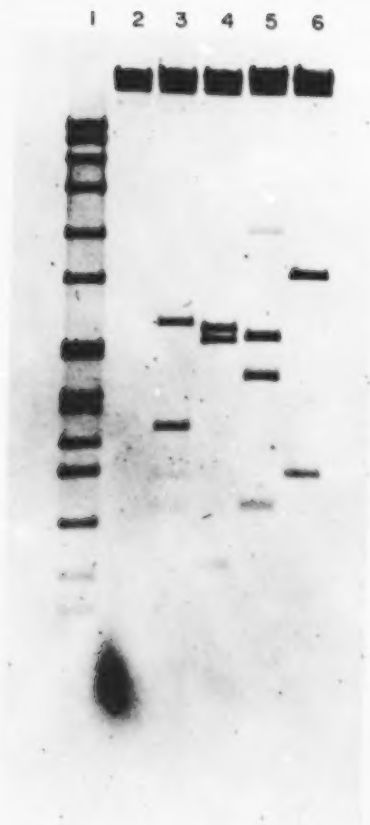


Figure 7. Restriction analysis of λgtll recombinant phage DNA. 1% agarose gel showing the following phage DNA cleaved with EcoRI: Lanes 2, λgtll; 3, λgt-MR-3A; 4, λgt-MR-6A; 5, λgt-MR-7A and 6 λgt-MR-8A. Lane 1 contained an EcoRI digest of SPP1 bacteriophage DNA for molecular weight standards.

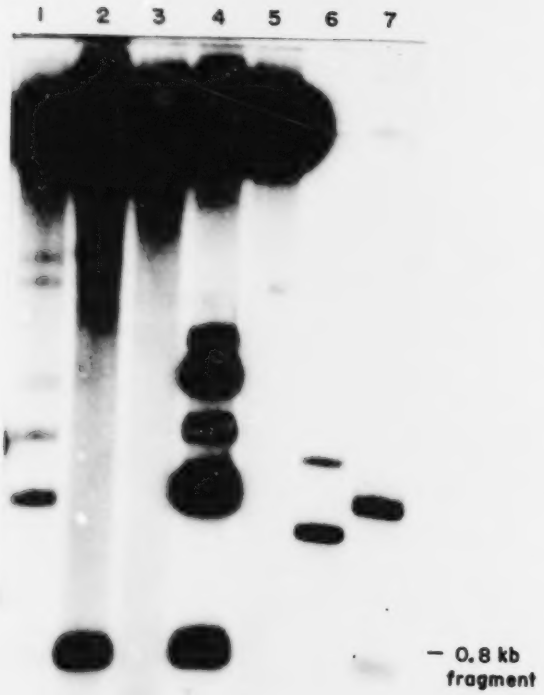


Figure 8. Autoradiograph of a Southern blot of λgt11 recombinant DNA probed with ^{32}P-labeled λgt-8γ DNA. The 0.7% agarose gel used for the blot contained the following DNA cleaved with the restriction enzymes(s) shown in parentheses: Lanes 1, λgt-8γ (EcoRI); 2, λgt-MR-3A (EcoRI); 3, λgt-MR-6A (EcoRI); 4, λgt-MR-7A (EcoRI); 5, λgt-MR-8A (EcoRI); 6, M. vannielii (HaeIII) and 7, M. vannielii (EcoRI). The position of the 0.8 kb EcoRI fragment in λgt-8γ, λgt-MR-3A, λgt-MR-7A and M. vannielii DNA that hybridized to the probe is indicated at the right.

methyl CoM-reductase genes even though they gave strong signals in the Ag:Ab screenings. For example, λgt-9γ DNA did not hybridize to either the 0.8 kb or 1.95 kb fragments of λ-8γ, even though both phages were identified with the anti-γ antisera probe. The possibility that λgt-9γ and λgt-8γ contained different portions of the γ-gene was eliminated when their DNA's failed to hybridize to common large restriction fragments in digests of M. vannielii genomic DNA. Similarly, analyses of the plasmids pUC8-γ1 to pUC8-γ4 showed that none of the cloned DNA fragments hybridized to each other or to any of the methanogen DNA's cloned in the λgtll recombinant phages. Furthermore, cloned M. vannielii DNA fragments in plasmids pUC8-α1, pUC8-α2 and pUC8-α3 did not cross hybridize with each other.

In view of the uncertainity as to which, if any, of the recombinant phages or plasmids contained methyl CoM-reductase genes, we decided to concentrate our efforts on analysing those phages which either contained common DNA fragments and/or were originally identified with the anti-γ antisera preparation, namely; λgt-8γ, λgt-9γ, λgt-MR-3A and λgt-MR-7A. First, we investigated whether the antigen(s) in lysates of λgt-MR-3A and λgt-MR-7A infected cells were also recognized by antibodies in the anti-γ sera. An appropriate dilution of phage was spotted onto a lawn of E. coli Y1090 (lon⁻) cells and nitrocellulose filters saturated with IPTG overlayed onto the surface of the plates. After incubation to allow the infected cells to lyse, the filters containing the bound lysate were removed and incubated with the anti γ, β or α antibody preparations. Immune complexes were detected with ^{125}I-protein A followed by autoradiography. Not suprisingly, it was found that the anti-γ sera which originally identified λgt-8γ and λgt-9γ as positive clones, also recognized λgt-MR-3A and λgt-MR-7A specified antigens; antibodies in the anti β and anti α sera preparations did not cross-react (Table 2). This result supported the conclusion that either the 0.8 kb EcoRI fragment common in λgt-8γ, λgt-MR-3A and λgt-MR-7A DNA or the 2.05 kb HaeIII fragment in λgt-9γ DNA was important in the expression of an antigen containing sequences of the γ-subunit polypeptide of methyl CoM-reductase. We also determined that expression of the antigen encoding gene was dependent on transcription initiated from the λgtll lacP promoter. An example of such an experiment, where synthesis of λgt-8γ and λgt-9γ specified antigen was compared in the presence and absence of IPTG, is shown in Figure 9. Based on this type of analysis it was clear that antigen synthesis in λgt-8γ, λgt-MR-3A and λgt-MR-7A infected E. coli cells was completely dependent on the functional activity of the lacP promoter of λgtll (Table 2).

To identify the antigen(s) reacting with antibodies in the anti-γ sera, polypeptides synthesized in phage infected minicells of E. coli were examined. Minicells are small anucleate cells formed by asymmetric cell division which have the capacity to transcribe and translate resident plasmid DNA templates or infected phage DNA's. Phage infected minicells were incubated in medium containing ^{35}S-methionine and the radio-actively labeled polypeptides subjected to SDS-PAGE analysis (Figure 10). In addition to the λ specified proteins of λgtll (lane 3), λgt-9γ and λgt-8γ infected minicells synthesized polypeptides of 12,000 and 70,000 daltons, respectively (lanes 1 and 2). Minicells infected with λgt-MR-3A synthesized four unique polypeptides of approximately 60,000, 55,000 17,000 and 10,000 daltons; λgt-MR-7A did not direct the synthesis of any novel polypeptides (Table 2). Polypeptides of similar molecular weight to the γ-subunit (approximately 35,000 daltons) were not detected among the

TABLE 2 CHARACTERIZATION OF POSITIVE CLONES IDENTIFIED WITH ANTI-γ ANTIBODIES

Phage	Size (kilobases) of cloned methanogen DNA excised with EcoRI	Size (daltons) of methanogen polypeptides synthesized in minicells	Dependence of Ag expression on IPTG	
			No IPTG	IPTG
λgt-8γ	0.8, 1.95	70,000	-	+
λgt-9γ	2.05	12,000	+	+
λgt-MR-3A	0.8, 0.9, 1.9, 2.1	60,000, 55,000 17,000, 10,000	-	+
λgt-MR-7A	0.8, 1.6, 1.95	-	-	+

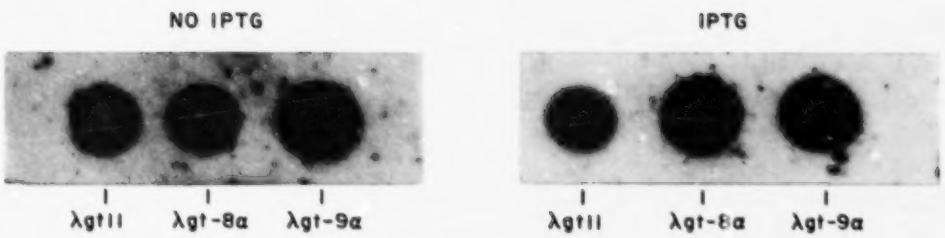


Figure 9. Effect of IPTG on the relative amount of antigen produced in cells infected with recombinant phage. Lysates from plaques (1cm) formed in the presence and absence of IPTG were lifted onto nitrocellulose and probed with anti-γ antibodies. The darkness of the lysed area indicates the amount of antigen present.

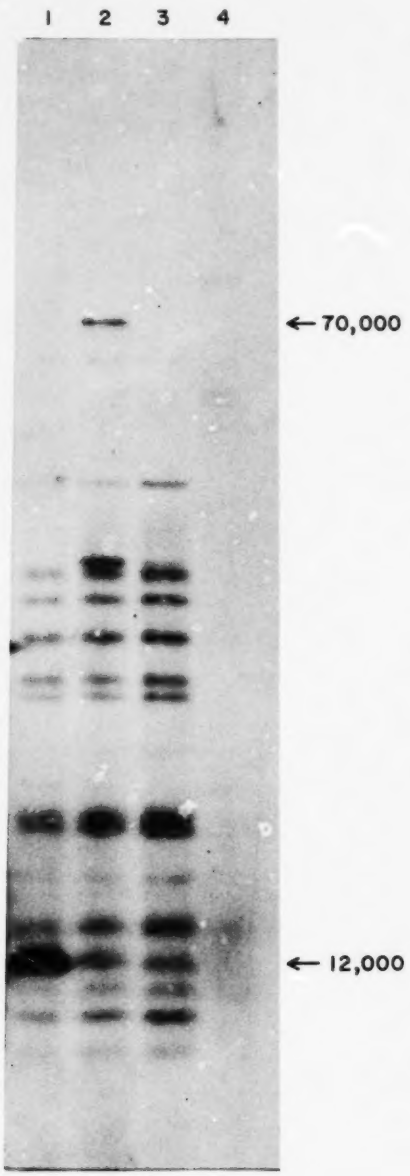


Figure 10. Analysis of polypeptides synthesized in λgt-8γ and λgt-9γ infected minicells. 10-20% gradient SDS-PAGE gel showing ^{35}S-methionine labeled polypeptides in minicells infected with the following phages: Lanes 1, λgt-9γ, 2, λgt-8γ, 3, λgtll and 4, no phage. The position and size (daltons) of unique methanogen polypeptides are indicated at the right.

polypeptides specified by the phage DNA's. The synthesis of different sized polypeptides in minicells infected with these phages was surprising in view of the fact that the phage DNA's (except for λgt-8γ)contained common restriction fragments (Table 2). However, it was possible that the antigen of interest might have been a large fusion polypeptide, containing part of the lacZ protein (β-galactosidase). Large polypeptides are frequently not detected using the minicell analysis as only very limited amounts of such polypeptides are synthesized in infected minicells.

To determine if any of the phage specified polypeptides produced in infected minicells were recognized by antibodies in the anti-γ sera, Western blotting analysis of infected cell lysates was undertaken. Cultures of the E. coli lon⁻ strain Y1090 were infected with the phages, IPTG was added and expression of phage DNA allowed to proceed for 45 minutes. Cells were pelleted and resuspended in a lysis buffer. Proteins in lysates were separated through SDS-PAGE, blotted onto nitrocellulose and incubated with the anti-γ sera and then with ^{125}I-protein A to detect Ag:Ab complexes. None of the polypeptides detected in minicells (Table 2) or any other high molecular weight polypeptides were recognized by anti-γ antibodies. Although many experiments were performed, varying the multiplicity of infection (0.5-10) and/or the time of phage DNA expression after infection (10-90 minutes) the antigens(s) which must have been present to give the positive signals in the plaque screenings, were never detected in gels. Even when E. coli strains lysogenized with the phage DNA's were employed to ensure that all cells in the population contained phage, the antigen(s) could not be identified. Since these negative results indicated that the antigen(s) was produced in extremely low amounts, we also attempted immune-precipitation of ^{35}S-labeled polypeptides from infected cell lysates as a means to identify the antigens. Protein A, linked to sepharose beads, was used to bind immune complexes formed. Surprisingly the analysis of dissociated immune precipitates on SDS-PAGE gels also failed to identify any radio-actively labeled antigens.

Although the antigen(s) could not be identified directly we decided that the 0.8 kb EcoRI fragment found in λgt-8γ , λgt-MR-3A and λgt-MR-7A DNA's was, in view of its frequent isolation, the most likely candidate to contain sequences of the γ-gene. This fragment would be insufficient in size to encode the complete γ subunit of methyl-CoM-reductase. A research group in Marburg (W. Germany) had already reported cloning the DNA encoding the γ and α genes of methyl CoM-reductase from the related organism M. voltae (19) and from our Southern blotting experiments, the 0.8 kb EcoRI fragment was highly conserved in the genome of M. voltae. This would be expected for a DNA sequence encoding what we had shown to be a highly conserved enzyme. We argued that if the sequence of DNA we had cloned from M. vannielii was similar to the methyl CoM-reductase genes cloned by the Marburg group we could conclude that we had cloned part of a methyl CoM-reductase gene from M. vannielii. The 0.8 kb EcoRI fragment was therefore cloned into pUC8 and sequenced by the chemical cleavage method of Maxam and Gilbert (20).

The DNA sequence we obtained (and the corresponding amino acid sequence) is shown in Figure 11. This sequence contains an open reading frame (122 codons) which must be the carboxyl terminus of a cloned gene unless an intron exists. Our antibody screenings indicated that this cloned DNA should be part of the

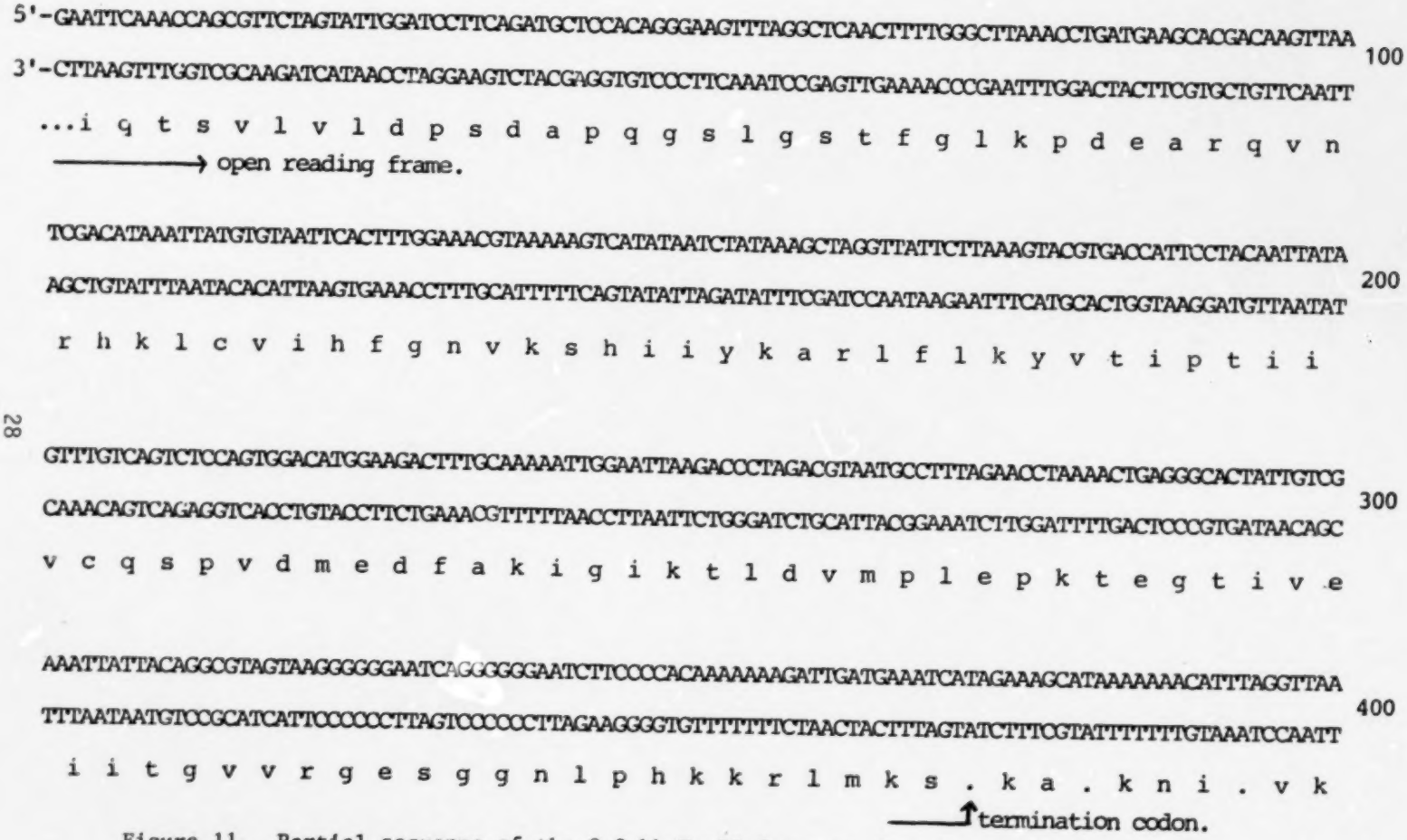

5'-GAATTCAAACCAGCGTTCTAGTATTGGATCCTTCAGATGCTCCACAGGGAAGTTTAGGCTCAACTTTTGGGCTTAAACCTGATGAAGCACGACAAGTTAA
3'-CTTAAGTTTGGTCGCAAGATCATAACCTAGGAAGTCTACGAGGTGTCCCTTCAAATCCGAGTTGAAAACCCGAATTTGGACTACTTCGTGCTGTTCAATT
...i q t s v l v l d p s d a p q g s l g s t f g l k p d e a r q v n
open reading frame.
TCGACATAAATTATGTGTAATTCACTTTGGAAACGTAAAAAGTCATATAATCTATAAAGCTAGGTTATTCTTAAAGTACGTGACCATTCCTACAATTATA
AGCTGTATTTAATACACATTAAGTGAAACCTTTGCATTTTTCAGTATATTAGATATTTCGATCCAATAAGAATTTCATGCACTGGTAAGGATGTTAATAT
r h k l c v i h f g n v k s h i i y k a r l f l k y v t i p t i i
GTTTGTCAGTCTCCAGTGGACATGGAAGACTTTGCAAAAATTGGAATTAAGACCCTAGACGTAATGCCTTTAGAACCTAAAACTGAGGGCACTATTGTCG
CAAACAGTCAGAGGTCACCTGTACCTTCTGAAACGTTTTTAACCTTAATTCTGGGATCTGCATTACGGAAATCTTGGATTTTGACTCCCGTGATAACAGC
v c q s p v d m e d f a k i g i k t l d v m p l e p k t e g t i v e
AAATTATTACAGGCGTAGTAAGGGGGGAATCAGGGGGGAATCTTCCCCACAAAAAAAGATTGATGAAATCATAGAAAGCATAAAAAAACATTTAGGTTAA
TTTAATAATGTCCGCATCATTCCCCCCTTAGTCCCCCCTTAGAAGGGGTGTTTTTTTCTAACTACTTTAGTATCTTTCGTATTTTTTTGTAAATCCAATT
i i t g v v r g e s g g n l p h k k r l m k s . k a . k n i . v k
termination codon.

Figure 11. Partial sequence of the 0.8 kb EcoRI fragment from M. vannielii DNA. The corresponding amino acid sequence with an open reading frame (122 codons) is shown below the DNA sequence. Phages containing the 0.8 kb fragment direct the synthesis of an antigen in E. coli which binds anti-γ antibodies.

gene encoding the γ subunit of methyl CoM-reductase. Comparison of this M. vannielii DNA sequence with the M. voltae sequence (personal communication, Dr. A. Klein, Marburg 6/85) indicated no similarity between our sequence and their sequence for the γ polypeptide of the M. voltae methyl CoM-reductase. Assuming that the Marburg group's cloned M. voltae sequences are correct we must conclude that the 0.8 kb EcoRI fragment of M. vannielii DNA does not contain any part of the gene encoding the γ polypeptide. Discussion of this anomolous and disturbing result with several investigators experienced in using the immunological screening procedure have elicited many descriptions of similar artifactually positive results. The general consensus appears to be that although this technique does allow the identification and isolation of desired genes it frequently requires extensive screening of positive clones before the correct positive is found. In order to conclusively identify a positive clone, a second independent screening procedure must be employed to sort through all the potential positive clones. One approach commonly used for this second screening employs a synthetic DNA probe designed from amino acid sequence data of the protein. Our current activity is aimed at purifying sufficient amounts of the three subunits of methyl CoM-reductase from M. vannielii to facilitate a biochemical determination of the sequence of amino acids at the amino termini of these subunits. If the amino acid sequences so obtained indicate that a specific DNA probe can be designed, it will be synthesized and used to screen our antigen positive clones.

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